Characterization of cadmium uptake, translocation and storage in near-isogenic lines of durum wheat that differ in grain cadmium concentration

Jonathan J. Hart[1,2], Ross M. Welch[1], Wendell A. Norvell[1] and Leon V. Kochian[1]

[1]US Plant, Soil and Nutrition Laboratory, United States Department of Agriculture, Agricultural Research Service, Ithaca, NY 14853, USA; [2]Department of Plant Biology, Cornell University, Ithaca, NY 14853, USA

Author for correspondence:
Jonathan J. Hart
Tel: +1 607 254 4919
Fax: +1 607 255 1132
Email: jjh16@cornell.edu

Received: *23 March 2006*
Accepted: *26 May 2006*

Summary

• Here we examined several physiological properties of two near-isogenic lines of durum wheat (*Triticum turgidum* var. *durum*) that differ in grain cadmium accumulation, to identify the function of a gene locus that confers differential grain Cd concentrations.
• Time- and concentration-dependent uptake and translocation studies using ^{109}Cd were conducted on nutrient solution-grown seedlings. Root extracts were analysed by inductively coupled plasma emission spectrometry, gel filtration and capillary electrophoresis to determine the interaction between Cd and phytochelatins (PCs) in storage of Cd in roots.
• The two isolines did not differ in time- or concentration-dependent root Cd uptake, but the low grain-Cd-accumulating isoline showed decreased movement of Cd from roots to shoots. All buffer-soluble Cd extracted from roots of both isolines was in the form of a low-molecular-weight PC-containing complex.
• The data suggest that PC synthesis is not a limiting factor in the differential storage of Cd in roots, and that movement of Cd through the root and into the transpiration stream may be the cause of differential Cd partitioning in the two isolines.

Key words: cadmium (Cd), *Triticum turgidum* var. *durum* (durum wheat), near-isogenic lines, partitioning, phytochelatins (PCs), root uptake, storage and translocation, zinc (Zn).

New Phytologist (2006) **172**: 261–271

doi: 10.1111/j.1469-8137.2006.01832.x

Introduction

Durum wheat (*Triticum turgidum* var. *durum*) is one of several crops that tend to accumulate relatively high concentrations of cadmium in plant tissues when grown in soils that contain elevated levels of that toxic metal. Because Cd represents a potential health threat to consumers, international trade organizations have sought to limit the acceptable concentration of Cd in edible crops sold in international markets. The Codex Alimentarius Commission (2005) has proposed maximum levels of 0.2 mg kg^{-1} Cd for wheat grain. Grain Cd concentrations in durum wheat grown in the Great Plains of North America, where soils can be naturally enriched in Cd from shale parent rock (Moran *et al.*, 1976), have been reported to exceed this concentration (Li *et al.*, 1997; Norvell *et al.*, 2000). Thus, to ensure the safety and marketability of durum wheat grown in these productive soils, it is important to elucidate the mechanisms controlling Cd accumulation in edible portions of crop plants.

To understand the physiology of Cd accumulation in durum wheat grain, it is helpful to trace the pathway of metal movement from soil to the reproductive parts of the plant. Because the chemical properties of zinc and Cd are similar, the pathway of Zn (an essential micronutrient) may serve as a predictive map for the movement of Cd within the plant. Uptake of Cd from soil solution occurs via a transport system in the

plasma membrane of root cells that normally functions to take up Zn (Hart *et al.*, 1998b; Hacisalihoglu *et al.*, 2001). Like Zn, Cd transport across the plasma membrane is a saturable process (Hart *et al.*, 1998a), and uptake competition between the two metal ions has been demonstrated (Hart *et al.*, 2002b). After it has been absorbed by root cells, Zn may be incorporated into enzymes and low-molecular-weight organic compounds (Longnecker & Robson, 1993). Substitution of Cd for Zn in these components can lead to phytotoxicity (Van Assche & Clijsters, 1990). Cadmium that is not bound or incorporated into cell constituents may be stored in root-cell vacuoles (Salt & Wagner, 1993; Ortiz *et al.*, 1995), or transported to shoots in the transpiration stream of the xylem (Jalil *et al.*, 1994; Salt *et al.*, 1995). Movement of Cd into developing grains occurs via phloem, probably by retranslocation from leaves and stems (Herren & Feller, 1997). This process can be inhibited by Zn (Herren & Feller, 1997), which exhibits a similar pattern of movement from vegetative to reproductive parts (Herren & Feller, 1996).

This study was designed to examine the potential role of root Cd uptake, translocation to the shoot, and storage in the root in the ultimate accumulation of Cd in durum wheat seed. Two near-isogenic lines of durum wheat that differ in grain Cd accumulation were grown in solution culture containing low concentrations of Cd to mimic the levels found in soil solution under field conditions. The results showed that differential grain Cd accumulation in the two isolines can be explained by differential Cd translocation to the shoot, and that the Cd remaining in roots is stored as a complex with phytochelatins.

Materials and Methods

Plant growth

The near-isogenic durum wheat lines W9262-339A-H (AH) and W9262-339A-L (AL) (Clarke *et al.*, 1997), which differ in grain Cd concentration, were used in these experiments. Plants were grown in solution culture as described previously (Hart *et al.*, 2005). Briefly, seeds were surface sterilized, imbibed overnight and placed on moistened filter paper in a Petri dish. Germinated seeds were transferred to polyethylene film cups and covered with polyethylene beads. Cups containing germinated seeds were positioned in holes cut into light-tight lids of 5-l pots filled with complete nutrient solution. The nutrient solution consisted of 1 mM KNO_3, 1 mM $Ca(NO_3)_2$, 50 μM $NH_4H_2PO_4$, 250 μM $MgSO_4$, 100 μM NH_4NO_3, 50 μM KCl, 12.5 μM H_3BO_3, 0.1 μM H_2MoO_4, 0.1 μM $NiSO_4$, 0.4 μM $MnSO_4$, 1.6 μM $CuSO_4$, 96 μM $Fe(NO_3)_3$, 118 μM *N*-(2-hydroxyethyl)ethylenediaminetriacetic acid (HEDTA), and 2 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer pH 6.0. Depending on the experiment, the nutrient solution also contained either 1 or 10 μM Zn-HEDTA, and either 0, 0.5 or 20 μM $CdSO_4$. The activities of Zn^{2+} and Cd^{2+} in nutrient solutions, as predicted by the chemical speciation software program GEOCHEM-PC (Parker *et al.*, 1995a) using equilibrium constants from Smith & Martell (1989), are listed in Table 1. HEDTA was included in the nutrient solution at concentrations approx. 20 μM higher than the total concentration of transition metal divalent cations. The HEDTA functioned to chelate micronutrient metals in solution and to buffer the chemical activities of free metal micronutrient cations (Norvell, 1991; Norvell & Welch, 1993; Parker *et al.*, 1995b). Pots containing seedlings were placed in a growth chamber with a photon flux density of 1000 μmol m^{-2} s^{-1} and a day : night temperature regime of 20 : 15°C with 16 h daylight. Solutions were replaced 12 d after transplanting to pots.

Table 1 Cd^{2+} and Zn^{2+} activities in nutrient solutions containing varying concentrations of Zn-HEDTA and $CdSO_4$

Concentration (μM)		Activity (nM)	
Zn	Cd	Zn^{2+}	Cd^{2+}
1	0	0.014	0
1	0.5	0.014	0.018
10	0.5	0.146	0.018
10	20	10.4	51.8

Additional *N*-(2-hydroxyethyl)ethylenediaminetriacetic acid (HEDTA) was supplied at a concentration approx. 20 μM higher than the total concentration of transition metal divalent cations. Free activities were calculated using GEOCHEM PC (Parker *et al*., 1995a).

Whole-plant analysis

At harvest, seedlings were rinsed in running 12-MΩ water, separated into roots and shoots by cutting 0.5 cm above and below the junction of root and shoot, and dried overnight in an oven at 60°C. Dried plant materials were weighed and digested, first in concentrated glass-distilled HNO_3 at 120°C, then in a 1 : 1 mixture of HNO_3 : $HClO_4$ at 160°C. The dry residue was dissolved in 1 ml 0.5 N HNO_3 and analysed for Cd and Zn via inductively coupled argon plasma emission spectrometry (ICP-ES; Model 1CAP 61E; Thermo-Jarrell Ash, Waltham, MA, USA).

Uptake and translocation experiments

Uptake experiments were performed as described previously (Hart *et al.*, 1998a). Briefly, roots of intact 8-d-old seedlings were rinsed in deionized water and placed in Plexiglas wells containing 60 ml aerated uptake solution consisting of 5 mM MES–Tris pH 6.0, 0.2 mM $CaSO_4$ and 12.5 μM H_3BO_3. Uptake experiments were initiated by addition of varying concentrations of ^{109}Cd-labeled $CdSO_4$ (3.7×10^4–1.2×10^6 Bq l^{-1}) to achieve Cd concentrations ranging from 25 nM

to 1.4 μM. In concentration-dependent uptake experiments, roots were exposed to radiolabeled Cd for 20 min. In time-dependent Cd-accumulation experiments, uptake solutions were refreshed at 20-min intervals as appropriate. Aliquots of uptake solutions taken at the beginning and end of each 20-min exposure interval were measured, and solution concentrations were calculated based on the average Cd concentration during each interval. Cadmium depletion from uptake solutions during the 20-min periods ranged from 18% in solutions containing 1.4 μM Cd to 70% at 26 nM Cd. At the end of the uptake period in both time- and concentration-dependent experiments, uptake solutions were withdrawn rapidly and replaced with a 2°C desorption solution containing 5 mM MES–Tris pH 6.0, 5 mM $CaSO_4$, 12.5 μM H_3BO_3, 0.15 nM $ZnSO_4$ and 100 μM nonradiolabeled $CdSO_4$ to remove loosely bound ^{109}Cd at the root surface. Following two 7.5-min desorptions, seedlings were removed from the wells and placed on damp paper towels. Roots were excised, weighed and analysed for ^{109}Cd using a gamma counter (model Auto-Gamma 5530, Packard Instruments, Meriden CT, USA).

In translocation and long-term Cd-accumulation experiments, roots of intact seedlings were immersed in an aerated solution containing 5 mM MES–Tris pH 6.0, 0.2 mM $CaSO_4$, 12.5 μM H_3BO_3, 7 μM EDTA and ^{109}Cd-labeled 0.5 μM $CdSO_4$ (1.5×10^5 Bq l^{-1}) in a 1-l Erlenmeyer flask. Excess EDTA (6.5 μM higher concentration than Cd) was included to buffer Cd^{2+} free activity at approx. 0.02 nM. Measurements of ^{109}Cd throughout the experiment showed little or no Cd depletion from uptake solutions. Experiments were carried out on a laboratory bench top with continuous unsupplemented ambient fluorescent lighting. After appropriate times up to 96 h, seedlings were transferred to uptake wells where roots were desorbed as described above. Roots and shoots were excised (approx. 0.5 cm above and below the root–shoot junction) and analysed for ^{109}Cd content. The results for the translocation experiments are presented in units of shoot Cd accumulation per total plant weight, to reflect the contributions of both roots and shoots in Cd translocation driven primarily by transpiration.

Root extractions

Cups containing 26-d-old plants grown in nutrient solution containing 1 μM Zn and 0.5 μM Cd were removed from pots and rinsed in running deionized water for 1 min. Cups were then placed in 5-l pots containing 12 MΩ water for 5 min, then placed in ice-cold 5 mM $CaCl_2$ for 10 min to remove root surface-bound Cd^{2+}, and returned to fresh 12 MΩ water (25°C) for 5 min. Roots were excised approx. 1 cm below the root–shoot junction, blotted on damp paper towel, weighed and placed in a −20°C freezer.

Frozen roots were ground in liquid N_2 with a mortar and pestle. Ground root material was transferred to a glass homogenizer, allowed to thaw, and ground again in 2 vol (w/v) homogenization buffer [50 mM Tris–HCl pH 8.6, 1 mM PMSF and 1% (v/v) Tween 20]. The homogenate was centrifuged at 48 000 ***g*** for 6 min and the supernatant was decanted and saved. The pellet was resuspended in homogenization buffer (same volume as first extraction), reground in glass homogenizer, and centrifuged again at 48 000 ***g***. Re-extraction and centrifugation was repeated a total of six times. Supernatants were combined and centrifuged at 100 000 ***g*** for 30 min. Pellets and combined supernatants were stored frozen at −20°C. Supernatants were cooled to −80°C, lyophilized and resolubilized in 18 MΩ water to a volume equivalent to the fresh weight of root at harvest. Pellets and a sample (2.15 ml) of resolubilized supernatant were analysed for metal content by ICP-ES. A second sample (2.15 ml) of resolubilized supernatant was injected onto a gel-filtration column containing Sephadex G-50, and chromatographed using a run buffer consisting of 40 mM KCl and 20 mM Tris–HCl. Run buffer was pumped through the column at a flow rate of approx. 1 ml min^{-1} at room temperature (25°C). Fractions ranging in volume from 4.7 to 5.9 ml were collected at 5-min intervals and the absorbance at 254 nm was recorded continuously. Samples (4.0 ml) were taken from fractions, dried and digested as described above for ICP-ES analysis. In some experiments, subsamples of 1.0–1.5 ml were taken from selected fractions and frozen at −80°C for later phytochelatin (PC) analysis.

Phytochelatin analysis

Samples obtained from gel-filtration fractions were lyophilized and 0.5 mg $NaBH_4$ (powder form) was added, followed by 98.4 μl 18 MΩ water and 1.6 μl 6 N HCl to lower the pH to approx. 2. Resolubilized samples were vortexed and placed on ice before analysis via capillary electrophoresis (CE) as described previously (Hart *et al.*, 2002a). Briefly, 20-μl samples were added to 30 μl 50 mM borate buffer pH 9.6, 10 μl 10 mM EDTA and 40 μl 1 mM 5-bromomethylfluorescein (Molecular Probes, Eugene, OR, USA), a thiol-reactive fluorescent tag. These 100-μl sample preparations were incubated in a water bath at 50°C for 20 min. A 50-μl aliquot was transferred to the CE sample holder for injection. The CE instrument was a Beckman P/ACE 5510 unit fitted with a 488 nm argon ion laser module. A 57-cm (50 cm from inlet to detector) fused silica capillary with 75-μm inside diameter was used. Running buffer was 50 mM borate pH 10.0. Prior to sample runs, the capillary was rinsed with 0.1 N NaOH for 15 min, then 18 MΩ water for 10 min. The protocol for each sample run was a 1-min prerinse with 18 MΩ water, then a 1-min rinse with running buffer followed by sample injection. Samples were pressure-injected (0.5 psi) for 5 s. Separation voltage was 30 kV with a 10-s ramp-up time. The capillary was temperature-controlled at 30°C. Electropherogram data were collected using P/ACE STATION (Beckman) software.

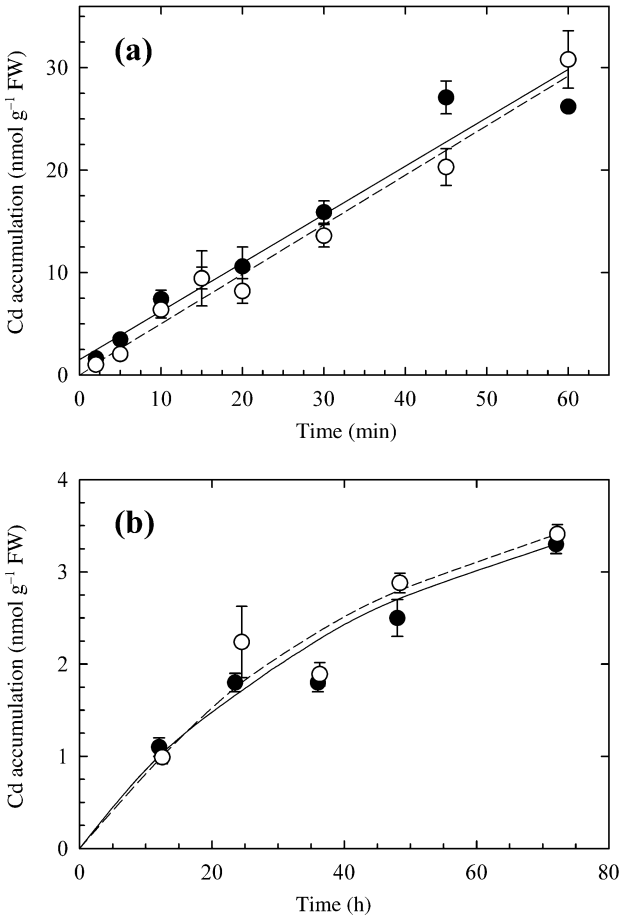

Fig. 1 Time-dependent accumulation of Cd^{2+} in intact seedling roots of low- (open symbols) and high-cadmium-accumulating (closed symbols) near-isogenic lines of durum wheat (*Triticum turgidum* var. *durum*). Roots were exposed to solutions containing 160 nM (free activity) ^{109}Cd (a) or 0.02 nM (free activity) ^{109}Cd (b) for the durations shown. Data points and error bars represent means ($n = 4$) and SEM. Error bars do not extend outside some data points.

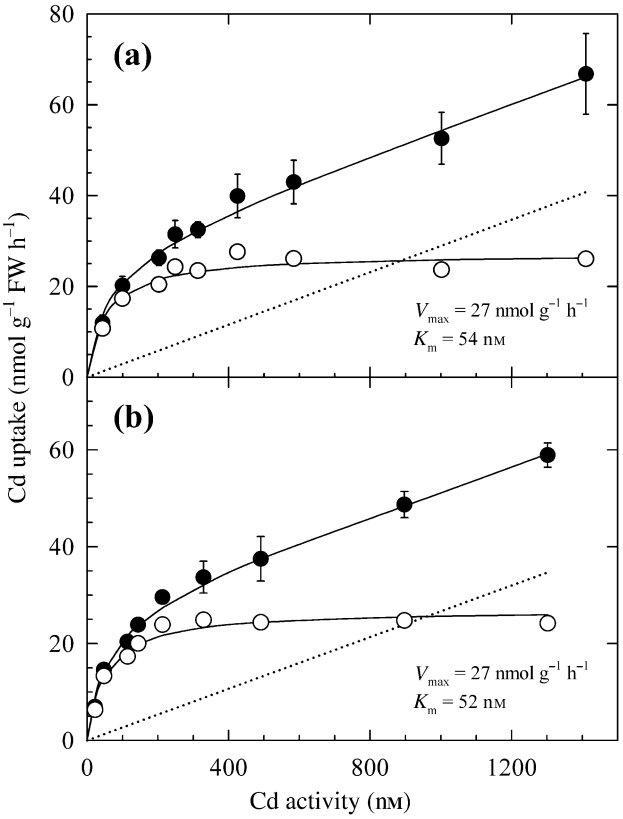

Fig. 2 Concentration-dependent $^{109}Cd^{2+}$ uptake in intact seedling roots of low- (a) and high-cadmium-accumulating (b) near-isogenic lines of durum wheat (*Triticum turgidum* var. *durum*). Linear (dotted line) and saturable (open symbols) components were derived from experimental data (closed symbols) by subtracting the equation for the regression line plotted through the five highest concentration data points. V_{max} and K_m values of saturable components were calculated by fitting a hyperbolic curve function to the saturable points. Data points and error bars represent means ($n = 4$) and SEM. Error bars do not extend outside some data points.

Statistical analysis

Where appropriate, differences between means were analysed using Student's *t*-test. Differences were considered significant when *P* values were < 0.01.

Results

Time- and concentration-dependent kinetics of root cadmium accumulation

The time-dependent kinetics of root Cd^{2+} accumulation in seedlings of both isolines was linear for at least the first 60 min of exposure to solutions containing 160 nM Cd^{2+} (Fig. 1a), a free activity higher than the K_m values derived in Fig. 2. Longer-term Cd^{2+} accumulation from a solution containing a free Cd^{2+} activity of 0.02 nM began to show saturation after approx. 24 h (Fig. 1b). The lower Cd^{2+} level was used in an effort to mimic field soil solution concentrations. No difference in Cd accumulation was observed between the two isolines in either experiment.

Concentration-dependent root unidirectional Cd^{2+} influx in both isolines was nonsaturating over a range of Cd^{2+} activities from 20 to 1400 nM (Fig. 2). Uptake curves were dissected mathematically into linear and saturable components. Previous experiments with durum wheat (Hart *et al.*, 1998a) showed that the linear component represented undesorbed binding of Cd^{2+} to the root apoplasm, while the saturable component corresponded to true influx of Cd^{2+} across the root-cell plasma membrane. In the experiments here, the linear components were derived from the five highest concentration data points and had slopes of 0.029 ($r^2 = 0.982$) and 0.027 ($r^2 = 0.999$) nmol g^{-1} FW nM^{-1} for the low- and high-Cd-accumulating isolines, respectively. The calculated saturable components of the two isolines had nearly identical kinetic constants (Fig. 2).

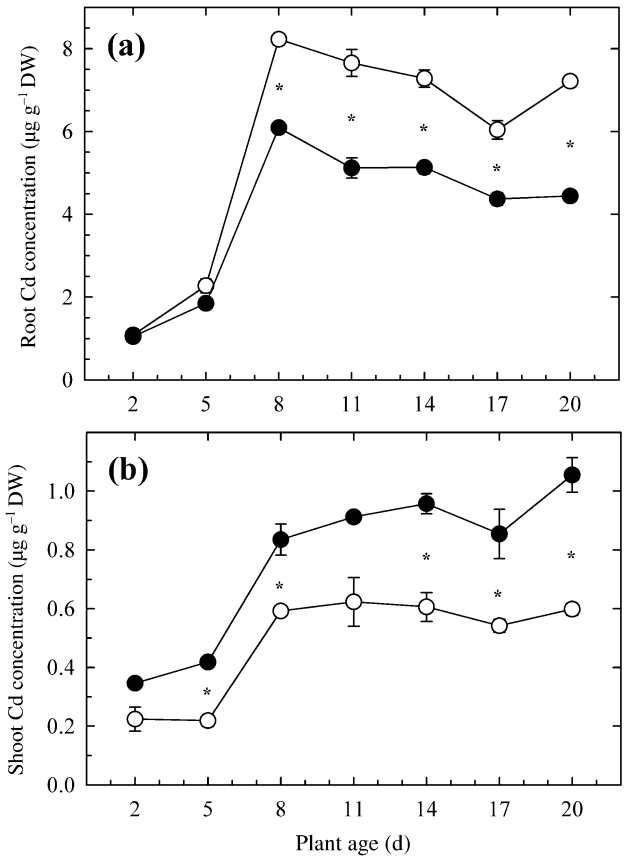

Fig. 3 Cadmium concentrations in roots (a) and shoots (b) of seedlings of low- (open symbols) and high-Cd-accumulating (closed symbols) near-isogenic lines of durum wheat (*Triticum turgidum* var. *durum*) through 20 d growth. Plants were grown in nutrient solution containing 0.5 μM Cd (0.02 nM free activity) and 10 μM Zn (0.15 nM free activity). Error bars represent the SEM of four replicates. Significant differences between isolines as determined by Student's *t*-test are indicated by * ($P = 0.01$).

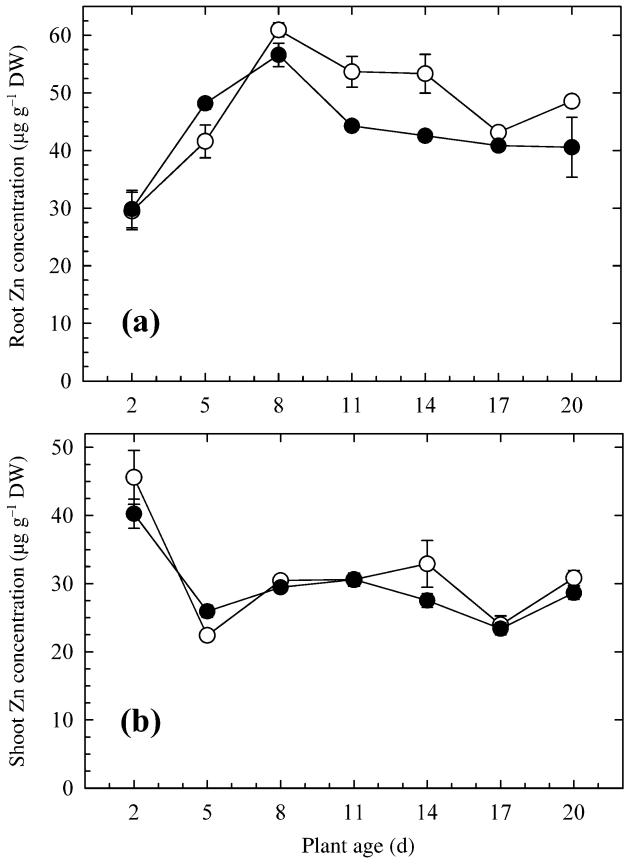

Fig. 4 Zinc concentrations in roots (a) and shoots (b) of seedlings of low- (open symbols) and high-cadmium-accumulating (closed symbols) near-isogenic lines of durum wheat (*Triticum turgidum* var. *durum*) through 20 d growth. Plants were grown in nutrient solution containing 0.5 μM Cd (0.02 nM free activity) and 10 μM Zn (0.15 nM free activity). Error bars represent the SEM of four replicates. There were no significant differences between isolines as determined by Student's *t*-test at $P = 0.01$.

Cadmium partitioning and translocation

In seedlings grown in nutrient solution containing total concentrations of 0.5 μM Cd and 10 μM Zn, which results in a free Cd^{2+} activity of 0.02 nM and a Zn^{2+} activity of 0.15 nM (Table 1), root Cd concentrations increased until day 8, then declined slightly through day 20 (Fig. 3a). Beginning on the fifth day and continuing through day 20, roots of the low-Cd-accumulating isoline seedlings had higher Cd concentrations than the high-Cd-accumulating isoline (Fig. 3a). Shoot Cd concentrations were higher in the high-Cd accumulator, beginning with the earliest measurement on day 2 and continuing at each measurement through day 20 (Fig. 3b). Cadmium concentrations in shoots generally continued to increase over the entire 20-d measurement period in the high-Cd-accumulating isoline, while they appeared to reach a maximum level in seedling shoots by day 11 in the low-Cd accumulator. In contrast to Cd, Zn concentrations in both roots and shoots differed little between isolines over the 20-d measurement period (Fig. 4). While the nutrient solution free activity of Zn was *c.* eight times higher than Cd (Table 1), Zn tissue concentrations were 8–30 times higher than Cd tissue concentrations in roots and 30–200 times higher in shoots.

Analysis of 24-d-old seedlings grown in 0.5 μM Cd and 1 μM Zn (see Table 1 for free activities), which were used for root extraction, showed a similar trend, but more marked differences in Cd distribution between roots and shoots (Table 2). Cadmium concentrations in shoots were more than threefold higher in the high- than in the low-Cd-accumulating isoline, while the mean Cd concentration in roots was lower (significant at $P = 0.06$, *t*-test). The resulting root : shoot ratio of Cd concentration was five times higher in the low- than in the high-Cd-accumulating isoline. The low-Cd-accumulating isoline also had lower total Cd concentrations in whole seedlings (roots and shoots combined).

Analysis of ^{109}Cd translocation from roots to shoots revealed that the two isolines began to show differences by

Parameter	Low-Cd accumulator	High-Cd accumulator
Cd concentration in root (μg g^{-1} FW)	0.382 (0.027)	0.265 (0.043)
Cd concentration in shoot (μg g^{-1} FW)	0.096 (0.007)*	0.330 (0.006)*
Root : shoot Cd concentration ratio	4.11 (0.56)*	0.814 (0.143)*
Cd concentration in seedling (μg g^{-1} FW)	0.250 (0.006)*	0.303 (0.032)*
Soluble Cd in LMW peak (%)	102 (3)	97 (6)
Insoluble Cd in extract (% of total Cd)	16 (2)	12 (1)

Values represent means (SEM) of four replications. Significant differences between isolines as determined by Student's *t*-test are indicated by * ($P = 0.01$).
HEDTA, *N*-(2-hydroxyethyl)ethylenediaminetriacetic acid; LMW, low molecular weight.

Table 2 Concentrations of cadmium in seedlings and root extracts of low- and high-Cd-accumulating isolines of durum wheat (*Triticum turgidum* var. *durum*) grown for 24 d in nutrient solutions supplied with 0.5 μM Cd-HEDTA (0.02 nM free Cd^{2+} activity)

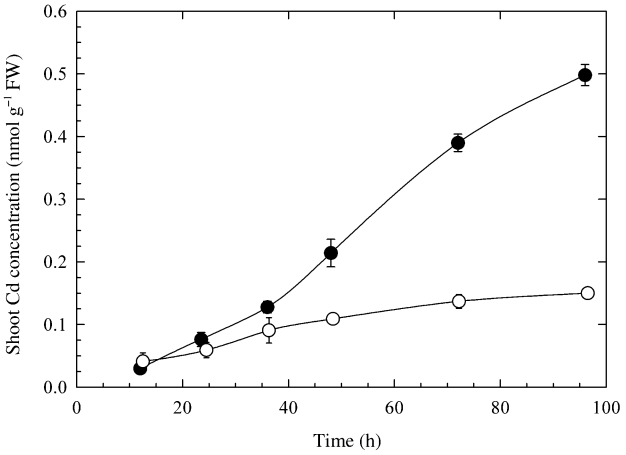

Fig. 5 Cadmium concentrations in shoots of seedlings of low- (open symbols) and high-Cd-accumulating (closed symbols) near-isogenic lines of durum wheat (*Triticum turgidum* var. *durum*). Intact seedling roots were immersed continuously in a solution containing 0.02 nM (free activity) ^{109}Cd for the length of time indicated, after which roots were desorbed with 100 μM Cd and shoots were analysed for ^{109}Cd. Results are presented in units of shoot Cd accumulation per total plant FW to reflect the contributions of both roots and shoots in transpiration-driven translocation. Data points and error bars represent means ($n = 4$) and SEM. Error bars do not extend outside some data points.

Table 3 Contents of PC_2 and cadmium in fractions shown in Fig. 7, obtained from gel filtration of extracts from 24-d-old seedling roots of a high-Cd-accumulating isoline of durum wheat (*Triticum turgidum* var. *durum*) grown in 0.5-μM Cd-HEDTA (0.02 nM Cd free activity)

Fraction number	nmol PC_2	nmol Cd	PC_2 : Cd	nmol Zn	PC_2 : Zn
51	0.493	0.206	2.4	2.63	0.19
53	0.969	0.447	2.2	2.76	0.35
55	0.812	0.257	3.2	1.12	0.74

PC, phytochelatin; HEDTA, *N*-(2-hydroxyethyl)ethylenediaminetriacetic acid.

36 h after continuous exposure to radiolabeled Cd, and that differential translocation became more pronounced at the later time points of the experiment (48–96 h Cd exposure; Fig. 5). Although both isolines showed a continuous increase in Cd movement to shoots, the increase in Cd accumulation in the high-Cd-accumulating isoline was considerably greater.

Cadmium storage

Fractionation of the soluble portion of root extracts by gel filtration revealed that Cd is contained in discrete peaks separated on the basis of molecular weight (Fig. 6). In plants grown in nutrient solution with relatively high Cd concentrations (20 μM; 52 nM free Cd^{2+} activity), two Cd-containing peaks were evident, with a lower molecular weight (LMW) peak containing quantitatively more Cd than a higher molecular weight (HMW) peak (Fig. 6a). When grown in solutions containing a lower Cd concentration (0.5 μM; 0.02 nM free Cd^{2+} activity), a single Cd-containing peak was observed and much lower Cd concentrations were measured in fractions of this peak (Fig. 6b) compared with fractions from roots grown under higher Cd concentrations (Fig. 6a). Sulfur was also present in both HMW and LMW peaks of plants grown under both Zn concentrations, and coincided with both Cd and Zn (Fig. 6a,b). Zinc was also present with Cd in fractions from the LMW peak in both treatments (Fig. 6a,b). The data presented in Fig. 6(a,b) are representative of several repeated gel filtration runs: chromatograms from the two isolines were similar. For both isolines, all the Cd in the soluble portion of root extracts from low-Cd-grown seedlings was contained in the LMW peak (Table 2). The insoluble portion (pellet) of root extracts contained 12–16% of the total cadmium in roots, with no statistically significant difference between isolines (Table 2).

Characterization of LMW cadmium-containing peak

In gel-filtration experiments, the LMW Cd-containing peak eluted later than the molecular weight marker bacitracin, indicating that Cd-containing complexes had a molecular weight < 1423 Da (Fig. 7a). Fractions collected

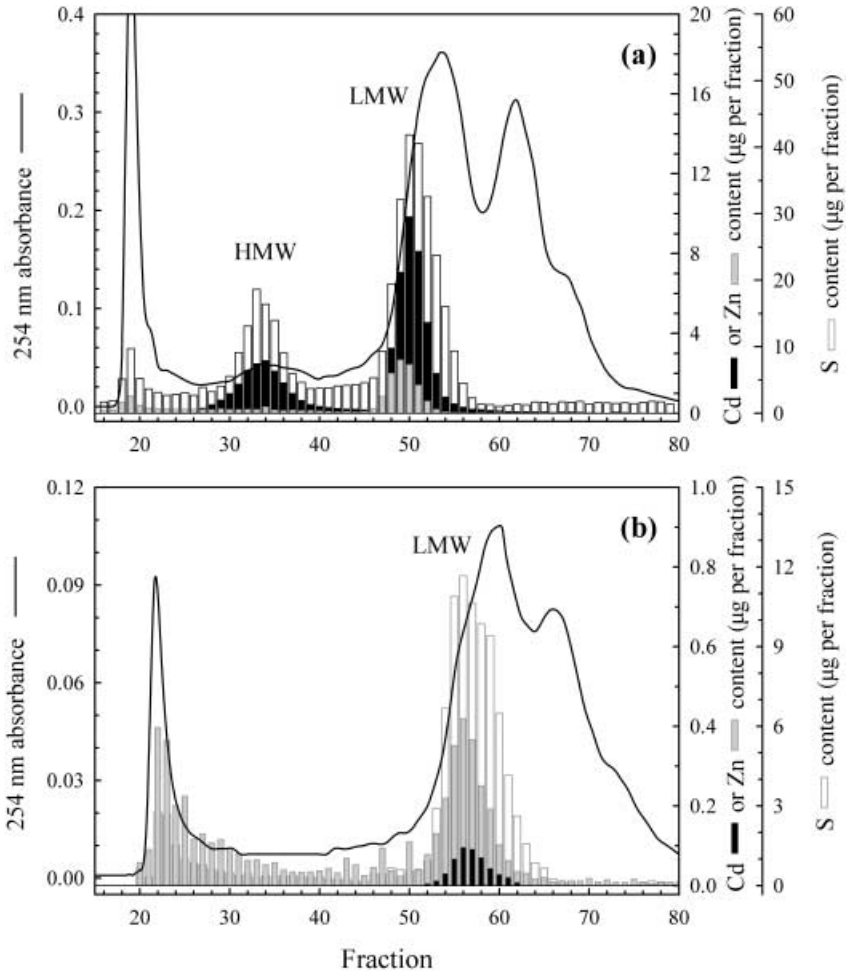

Fig. 6 Gel-filtration chromatography of buffer-soluble extracts of roots of a low-cadmium-accumulating isoline grown in 52 nM (free activity) Cd (a); and a high-Cd-accumulating isoline of durum wheat (*Triticum turgidum* var. *durum*) grown in 0.02 nM (free activity) Cd (b). Histograms show the content of Cd, zinc and sulfur in fractions of high-molecular-weight (HMW) and low-molecular-weight (LMW) peaks. Fractions volumes were 4.55 ml (a); 5.46 ml (b). The continuous trace represents A_{254}.

from gel-filtration runs were analysed by capillary electrophoresis and the resulting peaks were compared with PC and glutathione standards. Results showed that a peak corresponding to PC_2 (where PC_n refers to PC chain lengths of n repeating glutathione subunits) increased and decreased in magnitude in concert with Cd concentration in five fractions selected from the LMW peak (Fig. 7b–g). No PCs were detected in fractions outside the LMW peak. Glutathione, a precursor to PCs, exhibited the same pattern of concentration in the five fractions, while no peaks corresponding to PC_3 were detected in any of the five fractions. Comparisons of calculated concentrations of PC_2, Cd and Zn are shown in Table 3. The predicted molecular weight of a complex consisting of two PC_2 and two Cd moieties is approximately 1305, which is consistent with the elution of the LMW peak after bacitracin observed in Fig. 7(a).

Discussion

The use of near-isogenic lines of durum wheat that differ in grain Cd accumulation offers a means of deducing the function of a gene locus responsible for differential Cd accumulation. Considering that the differential Cd-accumulation trait in these isolines is likely to be controlled by a single gene or a group of closely linked genes, identification of differences in physiological responses in the two isolines may ultimately lead to knowledge of the function of the gene locus in question.

The absence of differences in time- or concentration-dependent Cd uptake between isolines (Figs 1, 2) shows that the low grain Cd trait is not directly related to root Cd uptake. Thus the gene responsible for low grain Cd is not likely to encode a protein associated with Cd influx across the plasma membrane. A lack of correlation between Cd root uptake and ultimate seed Cd concentrations has also been noted in other comparisons of durum wheat cultivars (Chan & Hale, 2004; Harris & Taylor, 2004).

Kinetic constants for Cd influx (Fig. 2) were similar to those reported previously for another durum wheat cultivar using the same technique (Hart *et al.*, 1998a). However, the kinetic constants differed from those reported by Harris & Taylor (2004), who reported much lower V_{max} values and higher K_m values in near-isogenic lines that are closely related

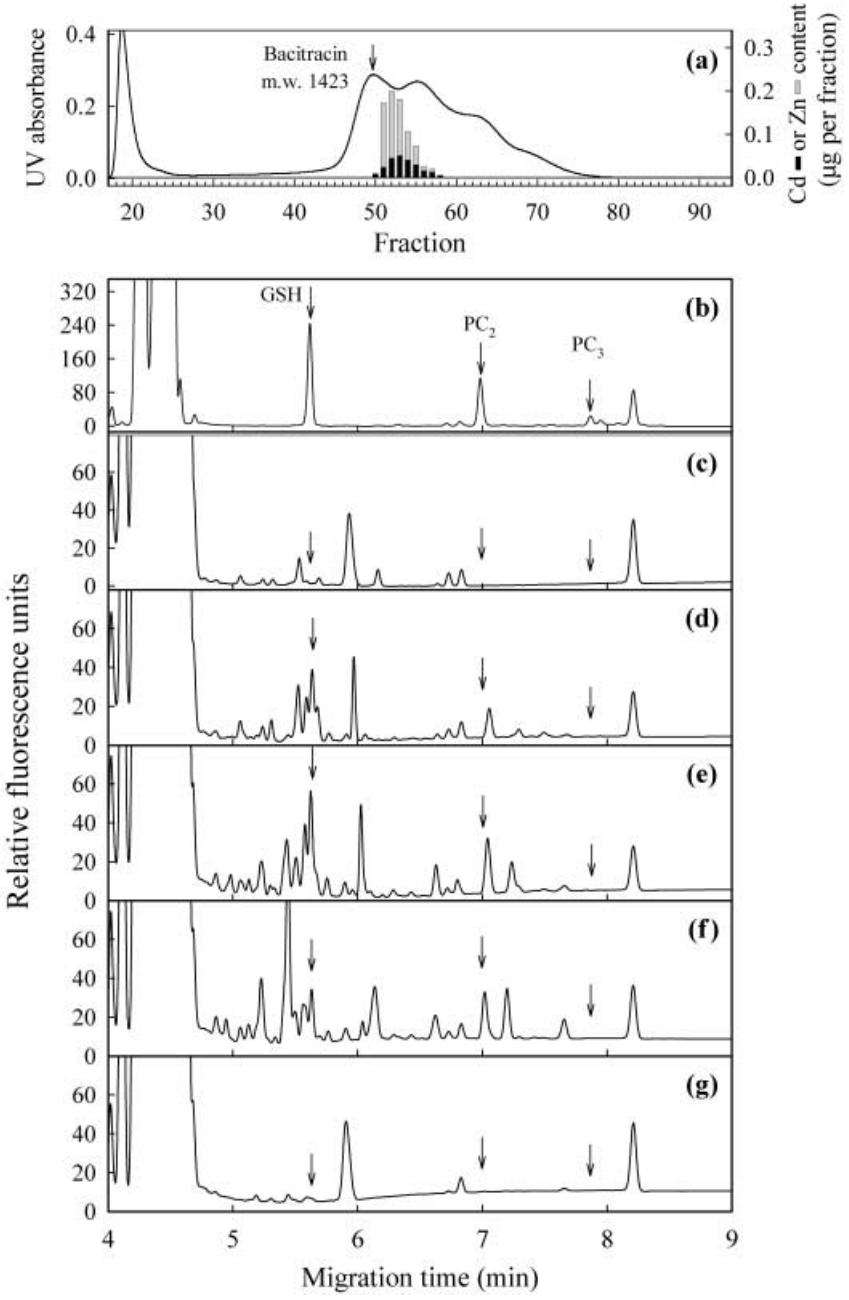

Fig. 7 (a) Gel-filtration profile of buffer-soluble extract from roots of a high-cadmium-accumulating isoline of durum wheat (*Triticum turgidum* var. *durum*) grown in 0.02 nM (free activity) Cd. Gel-filtration run included 12 mg bacitracin as a molecular weight marker. (b) Electropherogram of thiol standards. (c–g) Electropherograms of samples from gel-filtration fractions shown in (a): (c) fraction 43; (d) 51; (e) 53; (f) 55; (g) 63.

to those used in this study. It is possible that the discrepancy in kinetic constants is the result of differences in desorption techniques. In this and previous studies with Cd^{2+} and Zn^{2+} (Hart *et al.*, 1998a; 1998b), roots were desorbed with a relatively high concentration of nonradiolabeled metal, while Harris & Taylor (2004) used the metal chelator DTPA in their desorption regimen. DTPA has high-affinity constants for both Cd^{2+} and Zn^{2+}, and presents a risk for stripping Zn^{2+} from membranes, which may result in loss of membrane integrity (Welch & Norvell, 1993). The nearly linear Cd^{2+} uptake isotherms and very low rates of Cd^{2+} influx reported by Harris & Taylor (2004) suggest that some transporter-mediated Cd taken up previously may have been released by subsequent Cd leakage from root cells during the desorption process.

It is likely that differential partitioning of Cd between roots and shoots is the basis for ultimate differential grain Cd accumulation in the two isolines studied here. A similar conclusion was reached in studies of nonisogenic low- and high-Cd-accumulating durum wheat cultivars (Chan & Hale, 2004) and near-isogenic lines (Harris & Taylor, 2004), also grown in low, field-mimicking Cd concentrations. That shoot

Cd concentrations were different between isolines after only 36 h exposure to radiolabeled Cd (Fig. 5) suggests that differential movement of Cd from roots to shoots through the xylem was the fundamental process in differential root–shoot partitioning. The pattern of higher shoot Cd concentrations in the high-Cd-accumulating isoline throughout the first 20 d of growth (Fig. 3b), and subsequently through maturity in both vegetative and reproductive parts (Hart *et al.*, 2005), can be accounted for by differential root-to-shoot translocation through the xylem. One possible mechanism that could account for differential xylem translocation of Cd to shoots is the loading of Cd into xylem tissues for transport to shoots. It was recently reported that root-to-shoot translocation of Zn and Cd can be enhanced by overexpression of AtHMA4, a plasma-membrane heavy metal ATPase that mediates Zn and Cd efflux from plant cells and may be involved in xylem metal loading, in roots of *Arabidopsis thaliana* (Verret *et al.*, 2004). Possibly, a similar transporter in durum wheat may play a role in translocation of heavy metals to shoots, and that differential functioning or expression of such a transporter could account for differences in translocation of Cd to shoots in the two isolines studied here.

Although xylem transport of Cd to shoots is likely to be an important primary process underlying differential partitioning of Cd in the two isolines, retranslocation of Cd from shoots to roots via phloem probably also contributes to whole-plant Cd partitioning. Retranslocation to roots has been observed in other studies with Cd in durum wheat (Harris & Taylor, 2001) and other wheat genotypes (Cakmak *et al.*, 2000). Cadmium translocation through the phloem has also been shown to occur in movement from one isolated root section to another (Welch *et al.*, 1999), as well as in loading of seeds (Herren & Feller, 1997). In both cases, the presence of Zn inhibited Cd translocation. In this study, the higher root : shoot Cd ratio in plants grown in nutrient solutions containing low Zn concentrations (Table 2 vs. Fig. 3) suggests that Zn influenced xylem translocation from roots to shoots, possibly at the point of xylem loading. Herren & Feller (1997) showed that when excised shoots were placed in solutions containing Cd, Zn did not inhibit Cd movement into leaves, suggesting that Zn is not effective in altering Cd movement to shoots when root cell membranes are absent. A previous study (Hart *et al.*, 2005), using the same isolines as this study, showed that increasing the level of Zn in nutrient solution can reduce grain Cd concentrations. In that study, decreased Cd uptake caused by competition for transport at the plasma membrane of root cells (Hart *et al.*, 2002b) was cited as the primary factor in determining ultimate grain Cd concentrations. However, it is also possible that differential rates of xylem loading in the two isolines may also play a role, and that Zn may also have inhibited loading of Cd into xylem. At any rate, the higher levels of Cd partitioned into shoots in the high-Cd-accumulating isoline represent the likely source of increased Cd concentrations loaded into seeds via the phloem.

Although a difference in shoot Cd concentrations between isolines appeared within 2 d after exposure to Cd (Fig. 3a), a difference in root Cd concentrations was not measured until at least 4 d after exposure (Fig. 3b). The consistently higher subsequent root Cd concentrations in the low-Cd-accumulating isoline probably came about because of the lower relative Cd translocation rate to shoots in this isoline (Fig. 5). While lower root-to-shoot translocation could have been caused by reduced Cd loading into the xylem, cellular sequestration of Cd in root cortical cells would also inhibit movement of Cd to the stele, resulting in less movement of Cd to shoots and greater Cd concentrations in roots.

The possibility was investigated that the low-Cd-accumulating isoline sequestered more Cd in the roots as PC complexes. It is apparent from the data in Figs 6, 7 and Table 2 that all soluble Cd in roots of plants of both isolines grown in low Cd concentrations is stored as a low-molecular-weight complex with PCs. Based on the relative molecular weight in comparison with bacitracin (Fig. 7a), it appears that much of the stored Cd may be in the form of $(PC2)_2Cd_2$, with a molecular weight of approx. 1305. Rauser (2003) reported that, in maize, the Cd-binding PCs in an LMW peak, similar to that shown in Fig. 6(b), consisted of a variety of small-chain-length $(\gamma\text{-glu-cys})_n$ peptides. Data from the same report (Rauser, 2003) showed that the LMW peak predominated in the earliest measurement after initial plant exposure to Cd; subsequently the size of the HMW peak increased relative to the LMW peak. In this work, only the LMW peak was seen in roots grown at low Cd concentrations (considerably lower Cd activity than those used by Rauser, 2003); at higher Cd concentrations both peaks were observed (Fig. 6a,b). This pattern suggests that components of the LMW peak are formed initially when Cd levels are low; when Cd concentrations increase, components of the HMW peak begin to form. This hypothesis is supported by the emergence of an HMW peak when plants were grown in higher levels of Cd (Fig. 6a).

Interestingly, the mechanism responsible for reduced Cd translocation to shoots appears to be specific for Cd; there was little difference between isolines in root or shoot Zn concentrations (Fig. 3). This pattern was also observed by Harris & Taylor (2004) in related durum wheat isolines, and suggests that the physiological mechanism underlying differential Cd partitioning affects Cd to a greater degree than Zn. It is noteworthy that PCs have been demonstrated to have a higher binding affinity for Cd than for Zn (Cruz *et al.*, 2005), which might suggest that differential synthesis of PCs in the two isolines could lead to differential sequestration of Cd in root cells. However, it appears that synthesis of PCs is not the limiting factor in the compartmentation of Cd in either isoline, as all soluble Cd in roots of both isolines was associated with the PC-containing LMW peak (Table 2). Furthermore, both isolines had the capacity to synthesize and store much higher concentrations of Cd-PC (in the form of both LMW and HMW complexes) when seedlings were grown on higher

Cd concentrations (Fig. 6a; data for high-Cd-accumulating isoline were similar). Although PC profiles for only the high-Cd-accumulating isoline are presented (Fig. 7), it is highly likely that the low-Cd-accumulating isoline exhibits a similar pattern. Thus it seems improbable that the gene locus associated with low grain Cd levels is involved with PC synthesis.

The coincidence of both Zn and S in the LMW Cd-containing peak (Fig. 6b) is also of interest. It is perhaps not surprising to find S in fractions that contain thiol-rich PCs, but the basis for the much higher contents of S compared with Cd is not obvious, nor is the fact that the highest concentrations of S in total soluble root extracts were found in fractions of the LMW peak. Sulfide has been shown to be associated with PC–Cd complexes (Speiser *et al.*, 1992; Rauser, 2000) and may represent some of the S measured in the LMW peak in this study. The electropherograms of fractions taken from an LMW peak (Fig. 7) show the presence of glutathione and other unidentified thiol-containing components, which may also contribute to the total S measured in these fractions.

Perhaps of greater interest are the relatively high levels of Zn in the same LMW fractions that contain Cd and PC (Fig. 6a,b). This pattern suggests that Zn, too, may have been associated with PCs in LMW complexes. Cruz *et al.* (2005) recently reported relative stability constants for Cd^{2+} and Zn^{2+} binding with PC_3, and although PC_3 apparently displayed a higher binding affinity for Cd than for Zn, complexing of PC with Zn was demonstrated. In the absence of Cd, or when Cd is present at very low levels, as in this study, it seems likely that PC–Zn complexes should be present in root cells. In fact, Zn : Cd molar ratios of 4.5–13 were measured in fractions from the LMW peak (Table 3), suggesting that more Zn than Cd may have been associated with PCs. These data suggest that PCs may play a role in binding, storage and cellular homeostasis of Zn as well as Cd.

The physiological mechanism responsible for differential movement of Cd from roots to shoots remains unclear. Results from this study show that Cd in roots is located in vacuoles as PC complexes in both isolines, suggesting that Cd vacuolar influx is not different in the two isolines. However, it is possible that movement of Cd out of vacuoles could differ between isolines and ultimately affect translocation of Cd to shoots. Although transport processes involved with vacuolar influx of Cd have been identified (Cobbett & Meagher, 2002), similar mechanisms that control movement of Cd outward across the tonoplast in higher plants are less well understood.

Loading of Cd into the root xylem could also account for differential translocation of Cd to shoots in the two isolines. The mechanism(s) responsible for loading of Cd into xylem have not been well characterized, but several transporters from the P-type family of plasma membrane ATPases have been implicated in the loading of Zn, Cd and other micronutrients and heavy metals into the xylem. In *Arabidopsis*, it has been shown that the heavy metal association (HMA) ATPase transporters HMA2 and HMA4 are localized to the root vascular tissue, and when both are knocked out, xylem loading of Zn and Cd is inhibited (Hussain *et al.*, 2004). Thus it is possible that differences in root Cd retention in the two isolines may be caused by a differential transport function of a transporter in the HMA subfamily that plays a role in metal loading into the xylem. Another possible difference in Cd translocation from the root could involve the transport of Cd-binding organic ligands into the xylem. It has been shown that the loading of nickel into the xylem can be enhanced by providing free histidine to roots in *Alyssum* and *Brassica* species (Kerkeb & Krämer, 2003), and also that in a Ni-hyperaccumulating ecotype of *Alyssum lesbiacum*, high levels of histidine in the xylem correlate with high Ni translocation to the shoot (Krämer *et al.*, 1996). For Cd, it is possible that a ligand such as nicotianamine, which has been implicated in xylem movement of metals (Pich *et al.*, 1994), could play a role in durum wheat.

Whatever mechanism is responsible for differential Cd translocation to shoots in these isolines, it is important to note that the effect is specific to Cd and not Zn (cf. Figs 3, 4). If a transporter gene is ultimately responsible for differential transmembrane movement of Cd, the difference must lie in the function of the transporter, not in differential gene expression. Perhaps the active site on a transporter is altered to allow interaction and movement of Cd but not Zn. Additional investigation into specific Cd fluxes within root cells and among root tissues will be required to achieve an understanding of the mechanism(s) underlying differential movement of Cd to shoots in the two isolines studied here.

Acknowledgements

We thank Randy Clark for expert technical assistance.

References

Cakmak I, Welch RM, Hart J, Norvell WA, Oztürk L, Kochian LV. 2000. Uptake and retranslocation of leaf-applied cadmium (^{109}Cd) in diploid, tetraploid and hexaploid wheats. *Journal of Experimental Botany* **51**: 221–226.

Chan DY, Hale BA. 2004. Differential accumulation of Cd in durum wheat cultivars: uptake and retranslocation as sources of variation. *Journal of Experimental Botany* **55**: 2571–2579.

Clarke JM, Leisle D, DePauw RM, Thiessen LL. 1997. Registration of five pairs of durum wheat genetic stocks near-isogenic for cadmium concentration. *Crop Science* **37**: 297.

Cobbett CS, Meagher RB. 2002. Arabidopsis and the genetic potential for the phytoremediation of toxic elemental and organic pollutants. In: Somerville CR, Meyerowitz EM, eds. *The Arabidopsis book.* Rockville, MD, USA: American Society of Plant Biologists. http://www.aspb.org/publications/arabidopsis

Codex Alimentarius Commission. 2005. *Joint FAO/WHO Food Standards Programme. Twenty-Eighth Session, Rome, Italy 4–9 July 2005.* Report of the 37th session of the Codex Committee on Food Additives and Contaminants, 25–29 April 2005. Para. 175, Appendix XXVI. The Hague, the Netherlands. http://www.codexalimentarius.net/web/reports.jsp]ALINORM 05/28/12

Cruz BH, Díaz-Cruz JM, Ariño C, Esteban M. 2005. Complexation of heavy metals by phytochelatins: voltammetric study of the binding of Cd^{2+} and Zn^{2+} ions by the phytochelatin $(\gamma\text{-Glu-Cys})_3Gly$ assisted by multivariate curve resolution. *Environmental Science and Technology* **39**: 778–786.

Hacisalihoglu G, Hart JJ, Kochian LV. 2001. High- and low-affinity zinc transport systems and their possible role in zinc efficiency in bread wheat. *Plant Physiology* **125**: 456–463.

Harris NS, Taylor GJ. 2001. Remobilization of cadmium in maturing shoots of near isogenic lines of durum wheat that differ in grain cadmium accumulation. *Journal of Experimental Botany* **52**: 1473–1481.

Harris NS, Taylor GJ. 2004. Cadmium uptake and translocation in seedlings of near isogenic lines of durum wheat that differ in grain Cd accumulation. *BMC Plant Biology* **4**: 4.

Hart JJ, Welch RM, Norvell WA, Sullivan LA, Kochian LV. 1998a. Characterization of cadmium binding, uptake, and translocation in intact seedlings of bread and durum wheat cultivars. *Plant Physiology* **116**: 1413–1420.

Hart JJ, Norvell WA, Welch RM, Sullivan LA, Kochian LV. 1998b. Characterization of Zn uptake, binding and translocation in intact seedlings of bread and durum wheat cultivars. *Plant Physiology* **118**: 219–226.

Hart JJ, Welch RM, Norvell WA, Kochian LV. 2002a. Measurement of thiol-containing amino acids and phytochelatin (PC_2) *via* capillary electrophoresis with laser-induced fluorescence detection. *Electrophoresis* **23**: 81–87.

Hart JJ, Welch RM, Norvell WA, Kochian LV. 2002b. Transport interactions between cadmium and zinc in roots of bread and durum wheat seedlings. *Physiologia Plantarum* **116**: 73–78.

Hart JJ, Welch RM, Norvell WA, Kochian LV. 2005. Zinc effects on cadmium accumulation and partitioning in near-isogenic lines of durum wheat that differ in grain cadmium accumulation. *New Phytologist* **167**: 391–401.

Herren T, Feller U. 1996. Effect of locally increased zinc contents on zinc transport from the flag leaf lamina to the maturing grains of wheat. *Journal of Plant Nutrition* **19**: 379–387.

Herren T, Feller U. 1997. Transport of cadmium via xylem and phloem in maturing wheat shoots: comparison with the translocation of zinc, strontium and rubidium. *Annals of Botany* **80**: 623–628.

Hussain D, Haydon MJ, Wang Y, Wong E, Sherson SM, Young J, Camakaris J, Harper JF, Cobbett CS. 2004. P-type ATPase heavy metal transporters with roles in essential zinc homeostasis in *Arabidopsis*. *Plant Cell* **16**: 1327–1339.

Jalil A, Selles F, Clarke JM. 1994. Effect of cadmium on growth and the uptake of cadmium and other elements by durum wheat. *Journal of Plant Nutrition* **17**: 1839–1858.

Kerkeb L, Krämer U. 2003. The role of free histidine in xylem loading of nickel in *Alyssum lesbiacum* and *Brassica juncea*. *Plant Physiology* **131**: 716–724.

Krämer U, Cotter-Howells JD, Charnock JM, Baker AJM, Smith JAC. 1996. Free histidine as a metal chelator in plants that accumulate nickel. *Nature* **379**: 635–638.

Li Y-M, Chaney RL, Schneiter AA, Miller JF, Elias EM, Hammond JJ. 1997. Screening for low grain cadmium phenotypes in sunflower, durum wheat and flax. *Euphytica* **94**: 23–30.

Longnecker NE, Robson AD. 1993. Distribution and transport of zinc in plants. In: Robson AD, ed. *Zinc in soils and plants*. Dordrecht, the Netherlands: Kluwer Academic, 79–91.

Moran SR, Arndt M, Bluemle JP, Camara M, Clayton L, Fenton MM, Harris KL, Hobbs HC, Keatinge R, Sackreiter DK, Salomon NL, Teller J. 1976. Quaternary stratigraphy and history of North Dakota, southern Manitoba, and northwestern Minnesota. In: Mahaney W, ed. *Quaternary stratigraphy of North America.* Stroudsburg, PA, USA: Dowden, Hutchinson & Ross, 133–158.

Norvell WA. 1991. Reactions of metal chelates in soils and nutrient solutions. In: Mortvedt JJ, ed. *Micronutrients in agriculture*. Madison, WI, USA: Soil Science Society of America, 187–227.

Norvell WA, Welch RM. 1993. Growth and nutrient uptake by barley (*Hordeum vulgare* L. cv. Herta): studies using an *N*-(2-hydroxyethyl) ethylenedinitrilotriacetic acid-buffered nutrient solution technique. I. Zinc ion requirements. *Plant Physiology* **101**: 619–625.

Norvell WA, Wu J, Hopkins DG, Welch RM. 2000. Association of cadmium in durum wheat grain with soil chloride and chelate-extractable soil cadmium. *Soil Science Society of America Journal* **64**: 2162–2168.

Ortiz DF, Ruscitti T, McCue KF, Ow DW. 1995. Transport of metal-binding peptides by HMT1, a fission yeast ABC-type vacuolar membrane protein. *Journal of Biological Chemistry* **270**: 4721–4728.

Parker DR, Norvell WA, Chaney RL. 1995a. GEOCHEM-PC. A chemical speciation program for IBM and compatible personal computers. In: Leoppert RH, Schwab AP, Goldberg S, eds. *Chemical equilibrium and reaction models*. Special publication no. 2. Madison, WI, USA: Soil Science Society of America, 253–269.

Parker DR, Chaney RL, Norvell WA. 1995b. *Chemical equilibrium models: applications to plant nutrition research*. In: Loeppert RH, Schwab AP, Goldberg S, eds. Soil Science Society of America Special Publications, 42. Madison, WI, USA: Soil Science Society of America, 163–200.

Pich A, Scholz G, Stephan UW. 1994. Iron-dependent changes of heavy metals, nicotianamine, and citrate in different plant organs and in the xylem exudate of two tomato genotypes. Nicotianamine as possible copper translocator. *Plant and Soil* **165**: 189–196.

Rauser WE. 2000. Roots of maize seedlings retain most of their cadmium through two complexes. *Journal of Plant Physiology* **156**: 545–551.

Rauser WE. 2003. Phytochelatin-based complexes bind various amounts of cadmium in maize seedlings depending on the time of exposure, the concentration of cadmium and the tissue. *New Phytologist* **158**: 269–278.

Salt DE, Wagner GJ. 1993. Cadmium transport across tonoplast of vesicles from oat roots. Evidence for a Cd^{2+}/H^+ antiport activity. *Journal of Biological Chemistry* **268**: 12297–12302.

Salt DE, Prince RC, Pickering IJ, Raskin I. 1995. Mechanisms of cadmium mobility and accumulation in Indian Mustard. *Plant Physiology* **109**: 1427–1433.

Smith RM, Martell AE. 1989. *Critical stability constants, Vol. 6: Second Supplement*. New York: Plenum Press.

Speiser DM, Abrahamson SL, Banuelos G, Ow DW. 1992. *Brassica juncea* produces a phytochelatin–cadmium–sulfide complex. *Plant Physiology* **99**: 817–821.

Van Assche F, Clijsters H. 1990. Effects of metals on enzyme activity in plants. *Plant, Cell & Environment* **13**: 195–206.

Verret F, Gravot A, Auroy P, Leonhardt N, David P, Nussaume L, Vavasseur A, Richard P. 2004. Overexpression of AtHMA4 enhances root-to-shoot translocation of zinc and cadmium and plant metal tolerance. *FEBS Letters* **576**: 306–312.

Welch RM, Norvell WA. 1993. Growth and nutrient uptake by barley (*Hordeum vulgare* L. cv. Herta): studies using an *N*-(2-hydroxyethyl) ethylenedinitrilotriacetic acid-buffered nutrient solution technique. II. Role of zinc in the uptake and root leakage of mineral nutrients. *Plant Physiology* **101**: 627–631.

Welch RM, Hart JJ, Norvell WA, Sullivan LA, Kochian LV. 1999. Effects of nutrient solution zinc activity on net uptake, translocation, and root export of cadmium and zinc by separated sections of intact durum wheat (*Triticum turgidum* L. var. *durum*) seedling roots. *Plant and Soil* **208**: 243–250.

